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Occurrence and Distribution of RFamide-Positive Neurons Within the Polyps of *Coryne* sp. (Hydrozoa, Corynidae)

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Abstract. The neuronal network of the hydrozoan polyp *Coryne* sp. contains RFamide-positive neurons. Within the body column, these neurons are centralized in a basal ring and a distal field of ganglionic cells surrounding the peristome. The capitate tentacles are traversed by thick RFamide-positive neurites. Their pericarya are centralized in the knobby heads of the tentacles, forming a brightly fluorescing plaque after immunolabeling with an antibody against the RFamide sequence. Numerous dendrite-like extensions project from these cells towards the cell bodies of the nematocytes. The possible role of these dendrites in communication between adjacent nematocytes is discussed.

Introduction

Cnidarian nematocytes are both sensory and effector cells that respond to adequate external stimulation with the discharge of their nematocysts. This extremely rapid, specialized exocytotic process is triggered by a combination of mechanical and chemical stimuli: a deflection or shift of the cnidocil within its stereovillar support has to be preceded or at least accompanied by the chemical stimulus (Pantin, 1942; Thurm and Lawonn, 1990; Brinkmann and Thurm, 1993).

Physiological experiments and *in vivo* observations of hydrozoan polyps—in most instances made on *Hydra*—revealed that the nematocytes of two individual polyps and even different nematocytes of the same polyp may respond to identical external stimulation with great statistical divergence, indicating the influence of additional external or intrinsic factors. Nematocyst discharge, for example, is affected by the duration of starvation, the contraction/relaxation state of the animal, or environmental conditions (Jones, 1947; Kass-Simon, 1988). Although some of these stimuli may be received by the nematocytes themselves or by adjacent epitheliomuscular cells (Watson and Hessinger, 1989), others are obviously acting on specialized sensory cells (Tardent and Schmid, 1972; Westfall and Kinnamon, 1978; Kinnamon and Westfall, 1982; Westfall and Rogers, 1990; Westfall *et al.*, 1991; Golz and Thurm, 1991, 1993).

Summation and integration of some of these sensory inputs are managed by a net of neurons (Josephson, 1974). The phylogenetically primitive nervous system consists of at least three types of neuronal cells: sensory cells, ganglionic cells, and morphologically distinguishable interneurons (Tardent and Weber, 1976). However, this strict classification has to be used carefully since one and the same neuronal cell may simultaneously be used for all three tasks (Westfall, 1973). The loosely organized neurons are interconnected to a functional network by mono- or bidirectional synapses (Westfall, 1970; Westfall *et al.*, 1971; Westfall and Kinnamon, 1984).

All neurons are located in the interstitial spaces of the epithelial cell layers. Although the sensory cells are usually arranged perpendicular to the mesoglea, most of the other neuronal cell types are situated parallel to it (*e.g.*, Westfall, 1988; Hobmayer *et al.*, 1990). Neurons and non-neuronal cells are in close spatial contact. They are functionally coupled by chemical synapses (Westfall and Kinnamon, 1984; Westfall, 1988). Thus, the occurrence of synapses between neurons and nematocytes (Westfall and Kinnamon, 1984) may indicate an efferent control of the nematocytes. To prove this assumption directly by electrophysiological measurements, our group established a new

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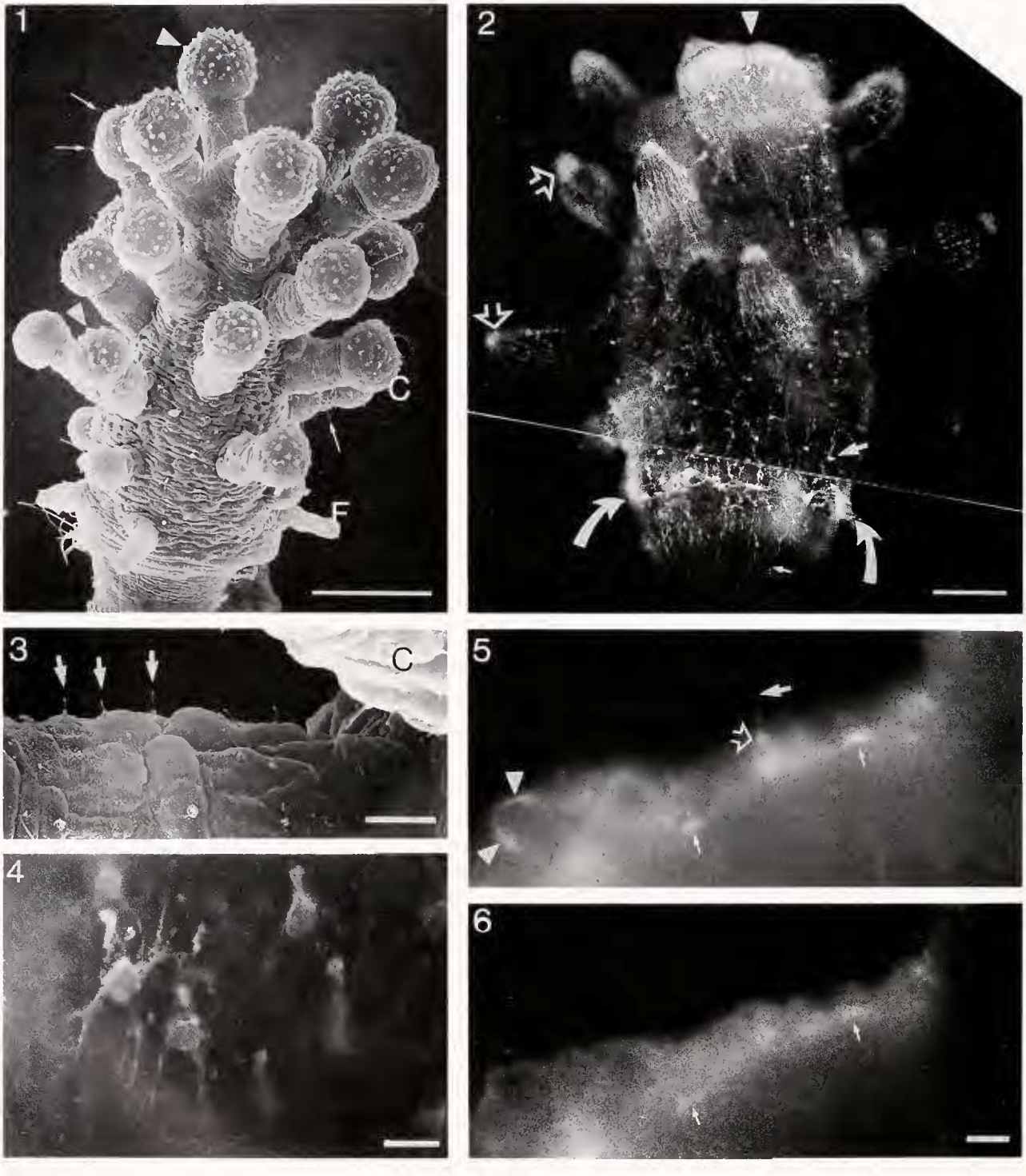


Figure 1. Scanning electron micrograph of *Coryne* sp. The body column of the polyp bears a whorl of filiform tentacles (F) and numerous capitate tentacles (C). Cnidocil complexes of nematocytes are marked by arrowheads; cilia of mechanosensory cells are labeled by arrows. Bar = 100 μm.

Figure 2. Fluorescence microscopical survey of *Coryne* labeled with anti-RFamide. A basal nerve ring (bent arrows), the heads of the capitate tentacles (open arrows), and a field of ganglionic cells around the peristome (arrowhead, *cf.* Fig. 4) are labeled. From the nerve ring some neurites (small arrow) branch off towards the stolon. Bright spots indicate the cell body of the neurons (arrow). Bar = 100 μm.

Figure 3. Scanning electron micrograph of a filiform tentacle. The cilia of the mechanosensory cells are marked by arrows. C = capitate tentacle of the next whorl. Bar = 10 μm.

model system: the marine hydrozoon *Coryne* sp. (Brinkmann and Thurm, 1991, 1993).

Like its close relative *Coryne pintneri* (*cf.* Stoessel and Tardent, 1971) *Coryne* sp. has hydranths that are characterized by a whorl of filiform tentacles and several rings of capitate tentacles. The latter are structurally differentiated into a short, contractile stalk and a knobby head containing many stenotele nematocytes.

The nematocytes of *Coryne* are amenable to intracellular electrophysiological recordings (Brinkmann and Thurm, 1993). To facilitate the interpretation of electrophysiological data, we started a parallel investigation of the neuronal organization of *Coryne* sp. by means of electron and fluorescence microscopy. To visualize the distribution of neurons, we used an antibody against neuropeptides of the RFamide type. Antibodies against the amidated carboxyterminus of these transmitter substances were produced by Grimmelikhuijzen and coworkers and have been successfully used for the study of cnidarian nervous systems by immunofluorescence and immunocytochemistry (for review see Grimmelikhuijzen *et al.*, 1991).

Materials and Methods

Coryne sp. was cultivated at the Zoological Institute of Münster in artificial seawater at about 14°C. The polyps were fed once a week with freshly hatched brine shrimps. All specimens are descendents of a single colony obtained from the Biological Station of Helgoland.

For electron microscopy, polyps were cut off from their stolons and transferred into artificial, calcium-free seawater supplemented up to 90 m*M* with $MgCl_2$ to prevent fixation-induced contractions. The polyps were fixed with 5% glutaraldehyde, 2% formaldehyde, 10% DMSO, 5 m*M* EGTA, 0.5% tannic acid in 0.1 *M* Na-cacodylate buffer (pH 7.4) for 30 min. The specimens were rinsed with 50 m*M* Na-cacodylate buffer (pH 7.4) and subsequently washed with the same buffer at pH 6.0. After postfixation with 5% tannic acid, 0.5% glutaraldehyde in 50 m*M* Na-cacodylate buffer (pH 6.0) for 15 min, the specimens were rinsed and stained with 1% OsO_4, 0.025% ruthenium red in 50 m*M* Na-cacodylate buffer (pH 6.0) for 5 min. During dehydration in a graded series of ethanol, the specimens were stained for 15 min by exposure to 1% uranyl acetate in 70% ethanol. The specimens were embedded in Spurr's resin following standard procedures. Ultrathin sections were made with a diamond knife on a MT 7000 microtome (Microm), poststained with lead citrate, and examined in a Philips EM 201.

Specimens used for scanning electron microscopy were fixed and dehydrated as described above. Then, the specimens were critical-point dried with carbon dioxide, sputtered with gold, and examined in a Hitachi EM S-530 at 25 kV.

For indirect immunofluorescence, the specimens were prepared using a procedure modified from Grimmelikhuijzen (1985). Polyps were fixed with 4% freshly prepared formaldehyde in phosphate buffered saline (PBS) for at least 12 h at 4°C. Then, the specimens were washed with PBS, incubated with 0.25% Triton X-100 in PBS for about 1 h, briefly washed with PBS, and rinsed with 0.4 *M* glycine, 1% goat normal serum in PBS for at least 4 h. The specimens were incubated at 4°C for about 12 h with the anti-RFamide antiserum 146III (kindly provided by Dr. Grimmelikhuijzen, Hamburg) diluted 1:100 with PBS. In double-labeling experiments, this solution was supplemented with a monoclonal antibody against β-tubulin (Sigma Chem.). After a short rinse with PBS, the specimens were washed with 0.4 *M* glycine, 1% goat normal serum in PBS for about 1 h. The binding of the RFamide-antibody was detected by labeling with FITC-conjugated anti-rabbit-IgG. The distribution of β-tubulin was viewed by labeling with TRITC-conjugated anti-mouse-IgG.

The specimens were examined in an Olympus IMT-microscope using 40×/0.5 or 60×/1.4-optics. Photographs of fluorescing specimens were made on TMAX-100 film exposed and developed as a 400-ASA film.

Results

In *Coryne* sp., the four filiform tentacles within the proximal whorl lack any nematocytes but contain numerous ciliated sensory cells (Figs. 1, 3). The number of whorls formed by capitate tentacles depends on the age of the polyps and reaches from one in newly outgrown animals to up to seven in older polyps. The knobby heads of these tentacles measure about 50 μm in diameter and contain numerous stenotele nematocytes (Figs. 1, 9) which are predominantly located within the upper two-thirds of the heads. Ciliated sensory cells of the same type as those of the filiform tentacles are not only integrated in the

Figure 4. Ganglionic cells stained by anti-RFamide. A group of these cells surround the peristom in a broad belt-like arrangement. Bar = 10 μm.

Figures 5–6. Double-labeled sensory cell in the stalk of a capitate tentacle. The antibody against β-tubulin stains the axoneme of the modified, immotile cilium (arrow in Fig. 5) and the microtubular cytoskeleton (open arrow) of the cell. The fluorescing microtubular basket of a nematocyte is marked by arrowheads. Some neurites (small arrows) are clearly visible. Only these are additionally stained by the anti-RFamide serum (small arrows in Fig. 6). Bar = 10 μm.

ectoderm of the stalks but also located between the nematocytes.

By incubation with the antibody against the RFamide, the polyps of *Coryne* sp. become intensely labeled, indicating the occurrence of RFamide-positive cells within their neuronal networks. Fluorescence microscopical surveys reveal the organization of this network (Fig. 2). The main part of the body column is covered by thin, brightly fluorescing strands that are only loosely interconnected by anastomosing fibers. Intensely fluorescing spots mark the pericarya of the neurons (arrows in Fig. 2). Two regions of the neuronal network are characteristically modified: a ring-like concentration of neurons surrounds the body column at the same level as the filiform tentacles; and groups of RFamide-positive cells are concentrated within the heads of the capitate tentacles. A brightly fluorescing patch around the peristome is formed by ganglionic cells (Figs. 2, 4). As indicated by double-labeling experiments with anti-tubulin and anti-RFamide, the sensory cells within the filiform tentacles do not contain RFamide-like peptides (Figs. 5, 6).

A great number of neurites within the body column originate from the basal nerve ring. From these nerve cells, thin neurites branch off towards the stolon and the peristome. Most of the basally directed neurites seem to terminate at the transition zone between body column and stolon, but a few neurones penetrate into the stolon (Fig. 2). However, the occurrence and distribution of RFamide-positive neurons within the stolons is difficult to determine by immunofluorescence, because the peridermal sheet not only hinders the penetration of antibodies into the tissue, but additionally complicates observations due to its autofluorescence. The distally oriented neurites participate in the formation of the neuronal network interconnecting its distal parts with the basal neuronal ring.

Numerous RFamide-positive cells elongate into the capitate tentacles. Immunofluorescence in the tentacles appears as arrays of thick (2–3 μm) fluorescent strands interspersed with occasional thinner ones (Fig. 7). Focus series revealed that the neurons are restricted to the ectodermal cell layer. The neuronal strands are situated immediately adjacent to the mesoglea. From these neurons only a few dendrite-like extensions project towards the surface of the stalks. However, these projections never correspond with the cytoplasm of the ectodermal ciliated sensory cells that are not stained by the antibody.

The pericarya of the nerve cells are concentrated within the center of the knobby heads (Fig. 8). Their intense fluorescence complicates the detection of subcellular details, but many thin dendrite-like extensions that grow out from the centralized cell bodies are clearly distinguishable from this background. A comparison of micrographs obtained by Nomarski interference contrast optics and fluorescence optics (Figs. 9, 10), reveals that the dendritic extensions project through the voluminous intercellular lumina towards the periphery of the ectodermal cell layer. Most of the dendrite-like extensions terminate near or at the cell bodies of the nematocytes, which themselves are not stained by the anti-RFamide. The second mechanosensitive cell type within the capitate tentacles, the sensory cells, are also not stained by this antibody, thus resembling their counterparts in the filiform tentacles.

Electron microscopical investigations confirmed that the capitate tentacles of *Coryne* sp. contain parallel bundles of neurons (Figs. 11, 12). These bundles are often composed of a central, thick neurite that is accompanied by a few thinner neurites. In cross-sections the thick neurites frequently appear to be encircled by thin protrusions of non-neuronal cells (Fig. 12). The protrusions contain myofilaments, an indication that they are formed by epitheliomuscular cells. Only the neurites are characterized by bundles of microtubules and contain aggregates of dense-cored vesicles with diameters of about 120 nm (Fig. 13). Besides the neurites, no other cellular compartments of the ectodermal cell layer contain similar amounts of microtubules. Thus, the fluorescence microscopical presentation of polymerized tubulin should reflect the distribution of neuronal cells within the tentacles of *Coryne*. By comparing this fluorescence pattern with the pattern obtained by the RFamide-antibody, the occurrence of neurons lacking RFamide-like peptides was investigated.

Double immunofluorescence experiments with anti-β-tubulin and anti-RFamide revealed that both antibodies produce identical staining patterns within the stalk of the tentacles (*cf.* Figs. 14, 15; 18, 19; and 20, 21) and within the filiform tentacles (*cf.* Figs. 16, 17). In the knob-like heads of the capitate tentacles, anti-β-tubulin additionally labels the microtubular cytoskeleton of the nematocytes. However, all neuron-shaped cells that are stained by the antibody against β-tubulin are simultaneously labeled by the anti-RFamide immunoglobulins. The only neuronal cells that contain a prominent microtubular cytoskeleton but lack RFamide-like immunoreactivity are the sensory cells of the filiform and capitate tentacles. Thus, the double-labeling experiments indicate that most of the neurons within the tentacles of *Coryne* sp. use RFamide-like peptides as neurotransmitters.

Discussion

RFamide-positive neurons have been identified in both polyps and medusae of numerous cnidarian organisms. Fluorescence microscopy has been used to examine the staining produced by specific antibodies against the carboxyterminus of RFamide-like neuropeptides and to demonstrate, thereby, the organization and development of the neuronal network in anthozoans (Grimmelikhuijzen *et al.*, 1989), scyphozoans (Anderson *et al.*, 1992)

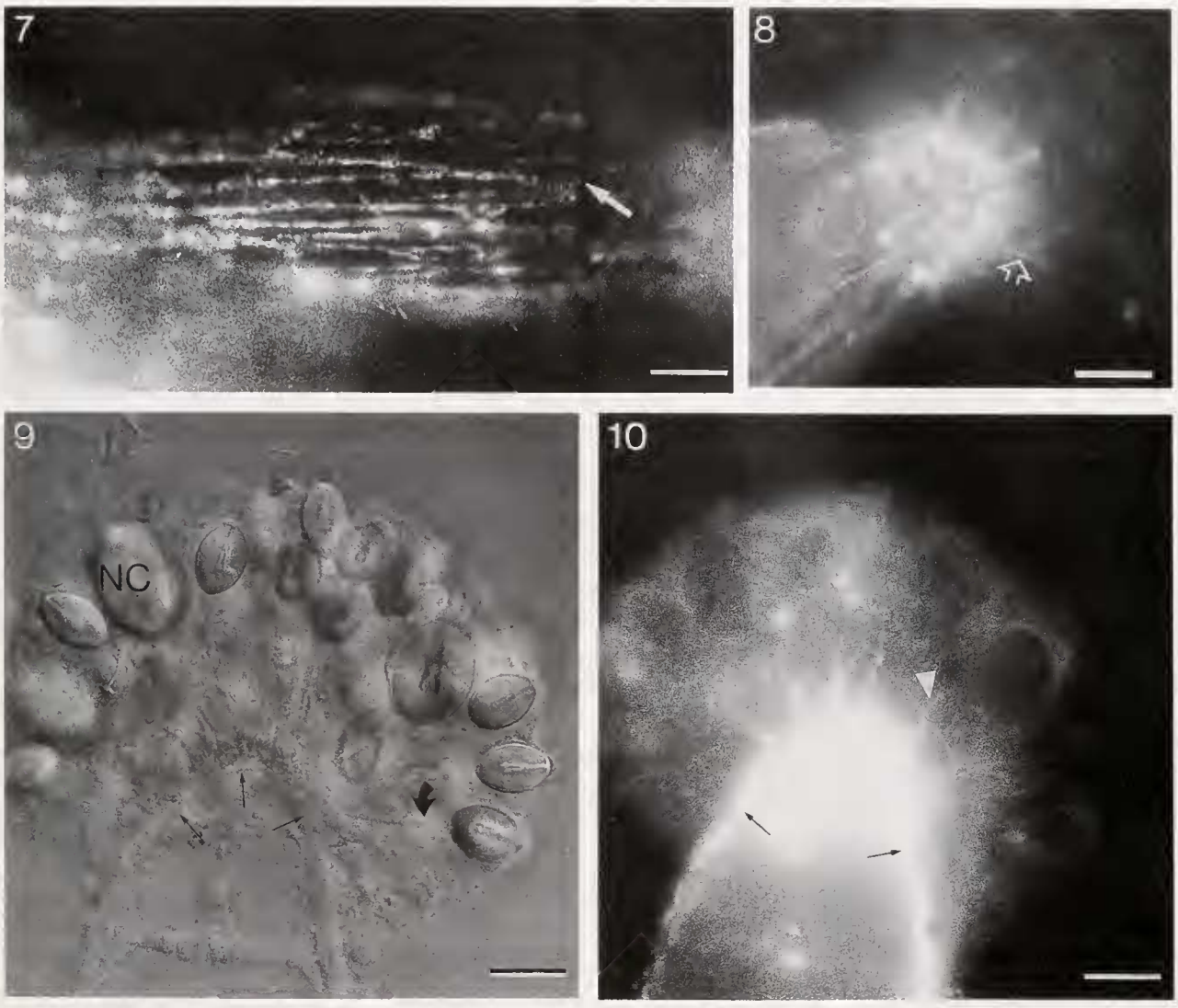


Figure 7. Stalk of a capitate tentacle. Thick parallel RFamide-positive neurites are linked by thin neurites (arrow). Bar = 20 μm.

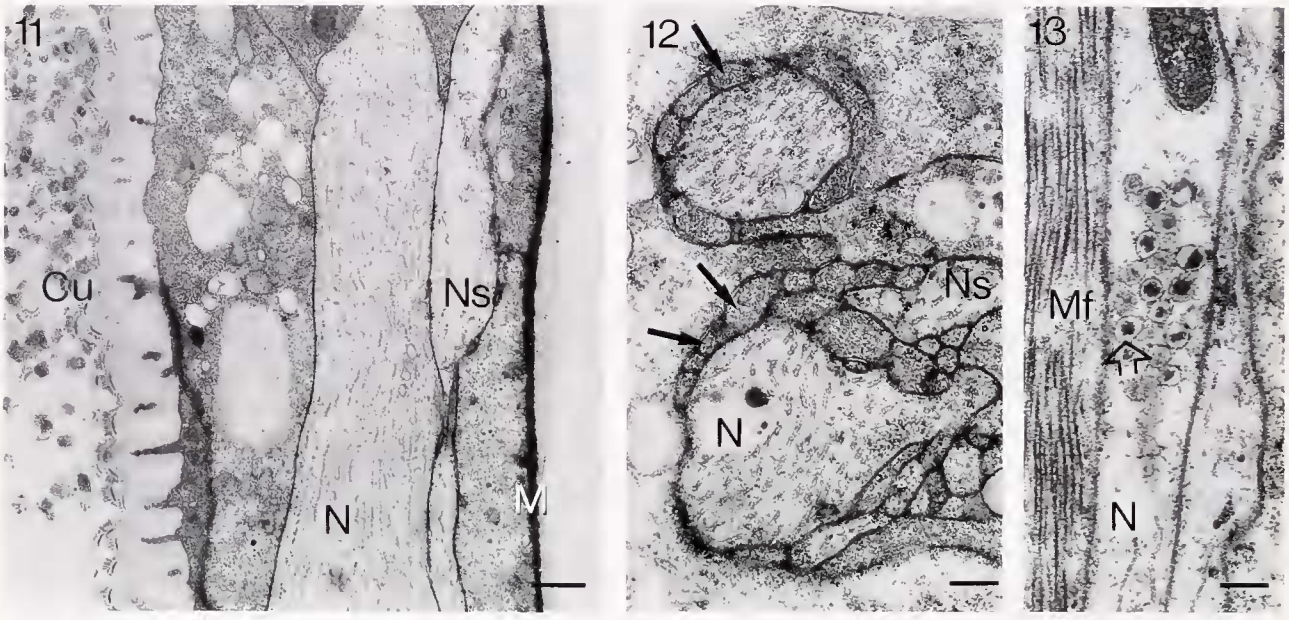
Figure 8. Head of a capitate tentacle focused on the mesoglea. The pericaryons of the neurites are centralized in the center of the heads. Thin dendritic protrusions (arrowhead) project towards the surface of the tentacle. Bar = 20 μm.

Figures 9–10. Nomarski interference contrast (Fig. 9) and fluorescence image (Fig. 10) of a capitate tentacle. The nematocytes (NC) are anchored at the mesoglea (small arrows) by thin, noncontractile stalks (bent arrow). Nematocytes and sensory cells are not labeled by the antibody against RFamides. RFamide-positive dendrites (arrowhead) terminate at the nematocytes. Bars = 20 μm.

and hydrozoans (Grimmelikhuijzen, 1985; for review see Grimmelikhuijzen *et al.*, 1991).

Although the occurrence of RFamide-like neuropeptides within the cnidarian nervous system is so far widely accepted, the use of nonpeptidic transmitters by cnidarians is still controversially discussed. Morphological, biochemical, and physiological data indicate that at least catecholamines may be additional candidates for neurotransmission (Wood and Lentz, 1964; Martin and Spencer, 1983; Kolberg and Martin, 1988; Takeda and Svendsen, 1991; Carlberg, 1992), but the cellular localization and distribution of these substances within the epithelial tissues and the neuronal network must be clarified more precisely.

In the present work, the antiserum 146111 against RFamides, which was produced by Grimmelikhuijzen and coworkers (for details see Grimmelikhuijzen, 1985), was shown to label the neuronal network of the hydrozoan polyp *Coryne* sp. Because the double-labeling experiments with anti-β-tubulin revealed that most of the



Figures 11–13. Ultrathin sections of neurites within the stalk. Thick neurites (N) are frequently accompanied by thinner neurites (Ns). All neurites are filled with densely packed microtubules. The thick nerve cells are often enclosed by thin non-neuronal protrusions (arrows in Fig. 12). Myofibrils of the epitheliomuscular cells are marked by Mf. The neurites contain dense-cored vesicles with diameters of approximately 120 nm (open arrow in Fig. 13). M = mesoglea, Cu = cuticula-like sheet around the tentacle. Bar in Figure 11 = 500 nm; in Figures 12 and 13 = 200 nm.

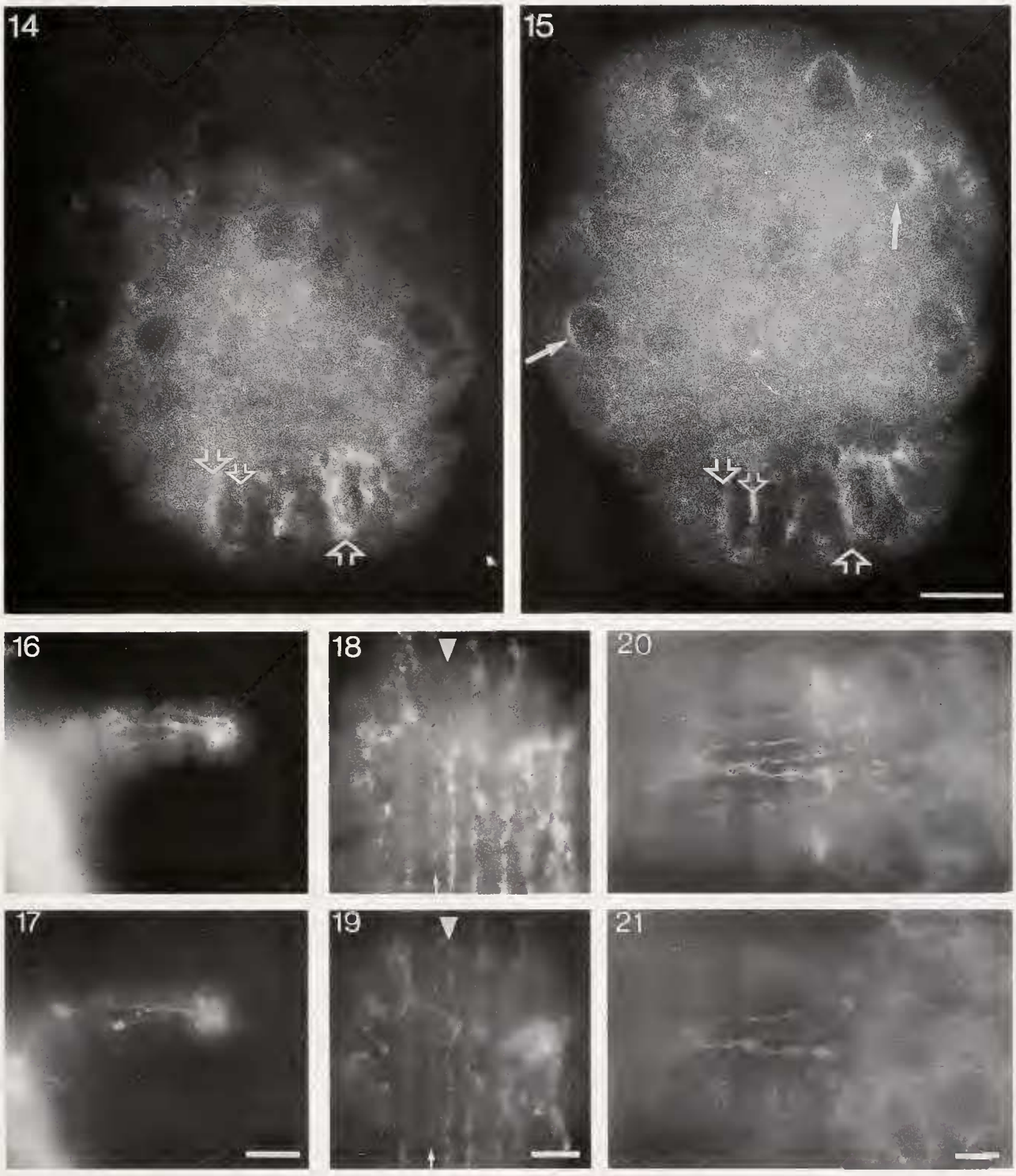
light microscopically detectable neurites are RFamide-positive cells, the obtained fluorescence patterns are thought to reflect the main portion of the neuronal system in *Coryne*.

The neuronal organization of *Coryne* closely resembles the neuronal networks of other hydrozoan polyps but also shows some specific structural adaptations. Prominent nerve rings like, for example, the basal one observed in *Coryne*, have been described for *Hydra oligactis*. In this organism, a prominent RFamide-positive nerve ring is located at the transition zone between body column and peristome (Grimmelikhuijzen, 1985; Koizumi *et al.*, 1992). Those regions of the body column from which whorls of tentacles emerge appear to be favored in the formation of nervous rings not only in hydrozoans but also in other cnidarians. Thus, the body column of the cubopolyp *Tripedalia*, for example, was also shown to be surrounded by a massive nervous ring at the level of the tentacular bases (Werner *et al.*, 1976).

Both organisms, *Coryne* sp. and its close relative *Coryne pintneri*, contain many mechanosensitive cells within their filiform tentacles (Tardent and Schmid, 1972). These cells are stimulated by water movements caused by prey organisms or experimental manipulations. After such an adequate stimulation, the polyp responds with a directed bending of its whole hydranth towards the stimulatory source (*cf.* Stoessel and Tardent, 1971). Thereby, the capitate tentacles are brought into the vicinity of a potential prey organism. Since, in *Coryne*, the ring-like centralization of neurons is only associated with the whorl of filiform tentacles, it may probably function as a first site of neuronal integration for incoming signals produced by the sensory cells within this type of tentacles. The coordination or activation of all muscles involved in the directed movements may be achieved by these neurons.

The ultrastructural investigations revealed that the neurons of *Coryne* contain numerous dense-cored vesicles. Koizumi *et al.* (1989) were able to demonstrate by immunocytochemistry that similar vesicles located within the epidermal ganglion cells in the peduncle of *Hydra* contain RFamide-like neuropeptides. Large numbers of these vesicles have been localized within the nerve terminals adjacent to the muscular base of the epitheliomuscular cells, indicating that this neuropeptide may be directly involved in neuromuscular transmission. That RFamides indeed have an excitatory effect on muscle and neuronal systems was demonstrated at least for sea anemones by McFarlane *et al.* (1987).

Although in other hydrozoan polyps, as for example *Hydra oligactis* and *Hydractinia echinata*, the pericarya of RFamide-positive neurons are homogeneously distributed over the entire length of their tentacles (Grimmelikhuijzen, 1985), the capitate tentacles of *Coryne* are characterized by a strong centralization of their RFamide-



Figures 14–15. Double-labeled capitate tentacle. Both figures have the same focal plane. The antibodies against RFamide (Fig. 14) and β-tubulin (Fig. 15) produce identical staining patterns within the stalks (indicated by open arrows). In the heads of the tentacles, only the microtubular baskets of the nematocytes (arrows) are labeled but no RFamide-based fluorescence occurs. Bar = 20 μm.

Figures 16–21. Pairs of double-labeled tentacle. Figures 16, 18, and 20 represent staining patterns produced by anti-β-tubulin; Figures 17, 19, and 21 show the corresponding patterns obtained by the antibody against RFamides. All tubulin-positive neurites within the filiform tentacles are also stained by anti-RFamide (Figs. 16, 17). Within the stalks of the capitate tentacles, both antibodies produce identical staining patterns in thinner (small arrows) and thicker neurites (arrowheads). Bar in Figure 17 = 20 μm; in Figures 19 and 21 = 10 μm.

positive pericarya. Intracellular electrophysiological recordings revealed that the nematocytes in the knobby heads of *Coryne* become postsynaptically depolarized by the mechanical stimulation of a second nematocyte located within the same tentacle (Brinkmann and Thurm, 1993). Although the discharge of nematocysts does not depend on the presence of nerve cells (Aerne *et al.*, 1991), its probability is affected by such neuronal interactions. The observed concentration of RFamide-positive cells within the center of the knobby heads may be a consequence of the accumulation of all nematocytes in a restricted domain on the tentacular surface. By this arrangement, an effective neuronal interaction between adjacent nematocytes may be achieved. Neuronal interactions between adjacent nematocytes are also discussed to occur in scyphomedusae via RFamide-positive neurons (Anderson *et al.*, 1992).

The sensory cells spaced between the nematocytes within the knobby heads are thought to act on adjacent nematocytes by neuronal interactions similar to those observed between adjacent nematocytes. However, both mechanosensitive cells obviously do not transmit their signals via RFamides.

In each head of the knobby tentacles, neurons and nematocytes seem to be structurally and functionally interconnected in the same way as the corresponding cells within the tentacles of *Hydra:* a distinct number of nematocytes is integrated within an epitheliomuscular cell, thus forming an individual battery complex (Hufnagel *et al.*, 1985). Each battery is usually accompanied by a sensory and a ganglionic cell. The latter cell is not only synaptically linked to the nematocytes of its own battery, but is additionally responsible for the excitation of adjacent epitheliomuscular cells and their batteries of nematocytes (Westfall, 1988; Hobmayer *et al.*, 1990). In analogy to this system, the nematocytes of each capitate tentacle are thought to represent a battery complex. The communication between adjacent batteries, *i.e.*, neighboring capitate tentacles, has then to be mediated by the parallel bundles of neurites within the tentacular stalks.

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